Standards for Plant Synthetic Biology: A Common Syntax for Exchange of DNA Parts

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Summary

Inventors in the field of mechanical and electronic engineering can access multitudes of components and, thanks to standardisation, parts from different manufacturers can be used in combination with each other. The introduction of BioBrick standards for the assembly of characterised DNA sequences was a landmark in microbial engineering, shaping the field of synthetic biology. Here, we describe a standard for Type IIS restriction endonuclease-mediated assembly, defining a common syntax of twelve fusion sites to enable the facile assembly of eukaryotic transcriptional units. This standard has been developed and agreed by representatives and leaders of the international plant science and synthetic biology communities, including inventors, developers and adopters of type IIS cloning methods. Our vision is of an extensive catalogue of standardised, characterised DNA parts that will accelerate plant bioengineering.

Introduction

The World Bank estimates that almost 40% of land mass is used for cultivation of crop, pasture or forage plants. Plants also underpin production of building and packing materials, medicines, paper and decorations, as well as food and fuel. Plant synthetic biology offers the means and opportunity to engineer plants and algae for new roles in our environment, to produce therapeutic compounds and to address global problems such as food insecurity and the contamination of ecosystems with agrochemicals and macronutrients. The adoption of assembly standards will greatly accelerate the pathway from product design to market, enabling the full potential of plant synthetic biology to be realised.

The standardisation of components, from screw threads to printed circuit boards, drives both the speed of innovation and the economy of production in mechanical and electronic engineering. Products as diverse as ink-jet printers and airplanes are designed and constructed from component parts and devices. Many of these components can be selected from libraries and catalogues of standard parts in which specifications and performance characteristics are described. The agreement and implementation of assembly standards that allow parts, even those from multiple manufacturers, to be assembled together has underpinned invention in these fields.

This conceptual model is the basis of synthetic biology, with the same ideal being applied to biological parts (DNA fragments) for the engineering of biological systems. The first widely-adopted biological standard was the BioBrick, for which sequences and performance data are stored in the Registry of Standard Biological Parts (Knight, 2003). BioBrick assembly standard 10 (BBF RFC 10) was the first biological assembly standard to be introduced. Its key feature is that the assembly reactions are idempotent: each reaction retains the key structural elements of the constituent parts so that resulting assemblies can be used as input in identical assembly processes (Knight, 2003; Shetty *et al.*, 2008). Over the years, several other BioBrick assembly standards have been developed that diminish some of the limitations of standard 10 (Phillips & Silver, 2006; Anderson *et al.*, 2010). Additionally, several alternative technologies have been developed that confer the ability to assemble multiple parts in a single reaction (Engler *et al.*, 2008; Gibson *et al.*, 2009; Quan & Tian, 2009; Li & Elledge, 2012; Kok *et al.*, 2014).

While overlap-dependent methods are powerful and generally result in 'scarless' assemblies, their lack of idempotency and the requirement for custom oligonucleotides and amplification of even well characterised standard parts for each new assembly are considerable drawbacks (Ellis *et al.*, 2011; Liu *et al.*, 2013; Patron, 2014). Assembly methods based on Type IIS restriction enzymes, known widely as Golden Gate cloning, are founded on standard parts that can be characterised, exchanged and assembled cheaply, easily, and in an automatable way without proprietary tools and reagents (Engler *et al.*, 2009, 2014; Sarrion-Perdigones *et al.*, 2011; Werner *et al.*, 2012).

Type IIS assembly methods have been widely adopted in plant research laboratories with many commonly used sequences being adapted for Type IIS assembly and subsequently published and shared through public plasmid repositories such as AddGene (Sarrion-Perdigones *et al.*, 2011; Weber *et al.*, 2011; Emami *et al.*, 2013; Lampropoulos *et al.*, 2013; Binder *et al.*, 2014; Engler *et al.*, 2014; Vafaee *et al.*, 2014). Type IIS assembly systems have also been adopted for the engineering of fungi (Terfrüchte *et al.*, 2014) and 'IP-Free' host expression systems have been developed for bacteria, mammals and yeast (Whitman *et al.*, 2013).

To reap the benefits of the exponential increase in genomic information, DNA assembly and bioengineering technologies, biological assembly standards must be agreed for multicellular eukaryotes. A standard for plants must be applicable to the diverse taxa that comprise Archaeplastida and also be capable of retaining the features that minimize the need to re-invent common steps such as transferring genetic material into plant genomes. In this letter, the authors of which include inventors, developers and adopters of Golden Gate cloning methods from multiple international institutions, we define a Type IIS genetic grammar for plants, extendible to all eukaryotes. This sets a consensus for establishing a common language across the plant field, putting in place the framework for a sequence and data repository for plant parts.

Golden Gate Cloning

Golden Gate cloning is based on Type IIS restriction enzymes and enables parallel assembly of multiple DNA parts in a one-pot, one-step reaction. Contrary to Type II restriction enzymes, Type IIS restriction enzymes recognise non-palindromic sequence motifs and cleave outside of their recognition site (**Figure 1A**). These features enable the production of user-defined overhangs on either strand, which in turn allow multiple parts to be assembled in a pre-determined order and orientation using only one restriction enzyme. Parts are released from their original plasmids and assembled into a new plasmid backbone in the same reaction, bypassing time-consuming steps such as custom primer design, PCR amplification and gel purification (**Figure 1B**).

The one-step digestion-ligation reaction can be performed with any collection of plasmid vectors and parts providing that:

(a) Parts are housed in plasmids flanked by a convergent pair of Type IIS recognition sequences
(b) The accepting plasmid has a divergent pair of recognition sequences for the same enzyme, between which the part or parts will be assembled
(c) The parts themselves, and all plasmid backbones, are otherwise free of recognition sites for this enzyme

(d) None of the parts are housed in a plasmid backbone with the same antibiotic resistance as the accepting plasmid into which parts will be assembled
(e) The overhangs created by digestion with the Type IIS restriction enzymes are unique and non-palindromic

To date, several laboratories have converted 'in-house' and previously published plasmids for use with Golden Gate cloning and have assigned compatible overhangs to standard elements such as promoters, coding sequences and terminators found in eukaryotic genes (Sarrion-Perdigones *et al.*, 2011; Weber *et al.*, 2011; Emami *et al.*, 2013; Lampropoulos *et al.*, 2013; Binder *et al.*, 2014; Engler *et al.*, 2014). The GoldenBraid2.0 (GB2.0) and Golden Gate Modular Cloning (MoClo) assembly standards, the main features of which are described below, are both widely used having been adopted by large communities of plant research laboratories such as the European Cooperation in Science and Technology (COST) network for plant metabolic engineering, the Engineering Nitrogen Symbiosis for Africa (ENSA) project, the C4 Rice project and the Realizing Increased Photosynthetic Activity (RIPE) project. MoClo and GB2.0 are largely, though not entirely, compatible. Other standards have been developed independently resulting in parts that are non-interchangeable with laboratories using MoClo or GB2.0. Even small variations prevent the exchange of parts and hinder the creation of a registry of standard, characterised, exchangeable parts for plants. The standard syntax defined below addresses these points, establishing a common grammar to enable the sharing of parts throughout the plant science community, whilst maintaining substantial compatibility with the most widely adopted Type IIS-based standards.

A Standard Type IIS Syntax for Plants

Plasmid backbones of standard parts

For sequences to be assembled reliably in a desired order and in a single step, all internal instances of the Type IIS restriction enzyme recognition sequence must be removed. The removal of such sites and the cloning into a compatible backbone, flanked by a convergent pair of Type IIS restriction enzyme recognition sequences, is described as 'domestication'. Assembly of standard parts into a complete transcriptional unit uses the enzyme *Bsa*I. Standard parts for plants must minimally,

therefore, be domesticated for *Bsa*I (Figure 2). Parts must also be housed in plasmid backbones that, apart from the convergent pair of *Bsa*I recognition sites flanking the part, are otherwise free from this motif. The plasmid backbone should also not contain bacterial resistance to ampicillin/carbenicillin or kanamycin as these are commonly utilised in the plasmids in which standard parts will be assembled into complete transcriptional units (e.g. Sarrion-Perdigones *et al.*, 2013; Engler *et al.*, 2014) (Figure 2). When released from its plasmid backbone by *Bsa*I, each part will contain specific, four-base-pair, 5' overhangs, known as fusion sites (Figure 2).

For assembly of transcriptional units into multi-gene constructs MoClo and GB2.0 require that parts are free of at least one other enzyme. MoClo uses *Bpi*I to assemble multiple transcriptional units in a single step, with subsequent assembly of larger constructs using *Bsa*I or *Bsm*BI. GB2.0 uses *Bsa*I and *Bsm*BI for iterative assembly of transcriptional units into multigene constructs (Figure 2). All three enzymes recognise six base-pair sequences (and produce four-base-pair 5' overhangs) therefore recognition sites are relatively rare. Compatibility with MoClo and GB2.0 multi-gene assemble plasmid systems can be obtained by domesticating *Bpi*I and *Bsm*BI as well as *Bsa*I recognition sequences (Figure 2).

Standard parts

A standard syntax for eukaryotic genes has been defined and twelve fusion points assigned (Figure 3). Such complexity allows for the complex and precise engineering of genes that is becoming increasingly important for plant synthetic biology. Standard parts are sequences that have been cloned into a compatible backbone (described above) and are flanked by a convergent pair of *Bsa*I recognition sequences and two of the defined fusion sites. The sequence can comprise just one of the ten defined parts of genetic syntax bounded by an adjacent pair of adjacent fusion sites. However, when the full level of complexity is unnecessary, or if particular functional elements such as N- or C-terminal tags are not required, standard parts can comprise sequences that span multiple fusion sites (Figure 3).

The sequences that comprise the fusion sites have been selected both for maximum compatibility in the one-step digestion-ligation reaction and to maximise biological functionality. The 5' non-transcribed region is separated into core, proximal and distal

promoter sequences, with the core region containing the transcriptional start site (TSS). The transcribed region is separated into coding parts and 5' and 3' untranslated parts. For maximum flexibility, an ATG codon for methionine is wholly or partially encoded into two fusion sites. The translated region, therefore, may be divided into three or four parts. The 3' non-translated region is followed by the 3' non-transcribed region, which contains the polyadenylation sequence (PAS). Amino acids coded by fusion sites within the coding region have been rationally selected: Neutral, non-polar amino acids, methionine and alanine, are encoded in the 3' overhangs of parts that may be used to house signal and transit peptides in order to prevent interference with recognition and cleavage. An alternative overhang, encoding a glycine, is also included to give greater flexibility for the fusion of non-cleaved coding parts. Serine, a small amino acid commonly used to link peptide and reporter tags, is encoded in the overhang that will fuse C terminal tag parts to coding sequences.

Universal acceptor plasmids

Universal acceptor plasmids (UAP) allow the conversion of any sequence to a standard part in a single step (Figure 4). This is achieved by polymerase chain reaction amplification of desired sequences as a single fragment or, if restriction sites need to be domesticated, as multiple fragments (Figure 4). The oligonucleotide primers used for amplification add 5' sequences to allow cloning into the UAP, add the standard fusion sites that the sequence will be flanked with when released from the UAP as a standard part with *Bsa*I and can also introduce mutations (Figure 4). Two UAPs, pUPD2 (https://gbcloning.org/feature/GB0307/) and pUAP1 (AddGene #71721) can be used to create new standard parts in the chloramphenicol resistant pSB1C3 backbone, in which the majority of BioBricks housed at the Registry of Standard Parts are cloned. A spectinomycin resistant UAP, pAGM9121 has been published previously (AddGene #52833 (Engler *et al.*, 2014)).

Compatibility with multigene assembly systems

Standard parts are assembled into transcriptional units in plasmid vectors that contain the features and sequences required for delivery to the cell, for example Left (LB) and Right Border (RB) sequences and an origin of replication for *Agrobacterium*-mediated delivery. Subsequently, transcriptional units can be assembled into multigene constructs in plasmid acceptors that also contain these features. It is

important that a standard Type IIS syntax be compatible with the plasmid vector systems that are in common use such as GB2.0 and MoClo while also allowing space for further innovation in Type IIS-mediated multigene assembly methodologies and the development of plasmid vectors with features required for delivery to other species and by other delivery methods. The definition of a standard Type IIS syntax for plants is therefore timely and will allow the growing plant synthetic biology community access to an already large library of standard parts.

Summary

Synthetic biology aims to simplify the process of designing, constructing and modifying complex biological systems. Plants provide an ideal chassis for synthetic biology, are amenable to genetic engineering and have relatively simple requirements for growth, (Cook *et al.*, 2014; Fesenko & Edwards, 2014). However, their eukaryotic gene structure and the methods commonly used for transferring DNA to their genomes demand specific plasmid vectors and a tailored assembly standard. Here, we have defined a Type IIS genetic syntax that employs the principles of part reusability and standardisation. The standard has also been submitted as a Request for Comments (BBF RFC 106) (Rutten *et al.*, 2015) at The BioBrick Foundation to facilitate iGEM teams working on plant chassis. Using the standards described here, new standard parts for plants can be produced and exchanged between laboratories enabling the facile construction of transcriptional units. We invite the plant science and synthetic biology communities to build on this work by adopting this standard to create a large repository of characterised standard parts for plants.

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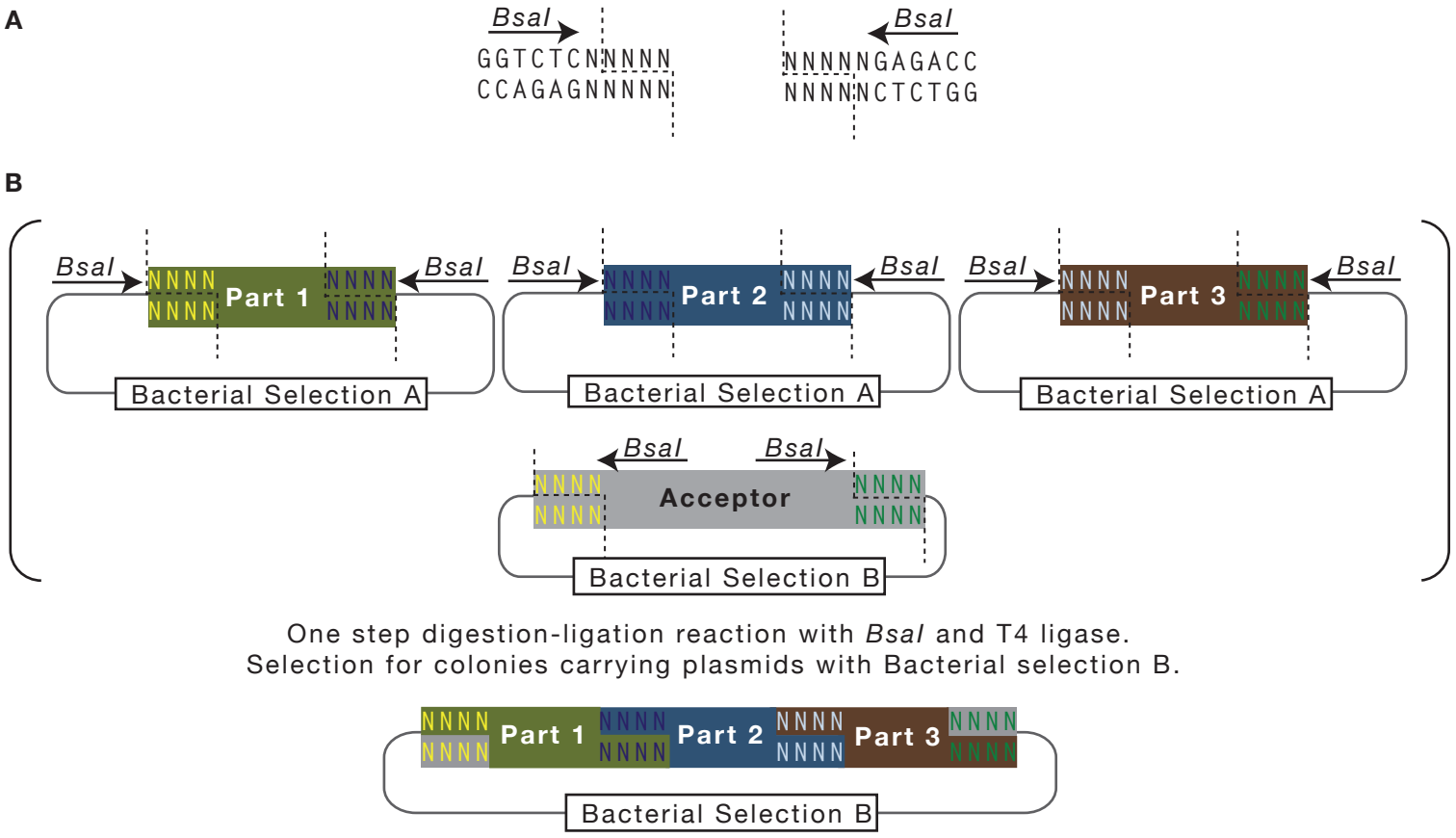
Figure 1 A Type IIS restriction enzymes such as *Bsa*I are directional, cleaving outside of their non-palindromic recognition sequences. **B** Providing compatible overhangs are produced on digestion, standard parts cloned in plasmid backbones flanked by a pair of convergent Type IIS restriction enzyme recognition sites can be assembled in a single digestion-ligation reaction into an acceptor plasmid with divergent Type IIS restriction enzyme recognition sites and a unique bacterial selection cassette.

Figure 2 A Standard parts for plants are free from *Bsa*I recognition sequences. To be compatible with Golden Gate Modular Cloning (MoClo) and GoldenBraid2.0 (GB2.0) they must also be free from *Bpi*I and *Bsm*BI recognition sequences. **B** Standard parts are housed in plasmid backbones flanked by convergent *Bsa*I recognition sequences. The plasmid backbones are otherwise free from *Bsa*I recognition sites. The plasmid backbone should not confer bacterial resistance to ampicillin, carbenicillin or kanamycin. When released from their backbone by *Bsa*I, parts are flanked by four-base-pair 5' overhangs, known as fusion sites.

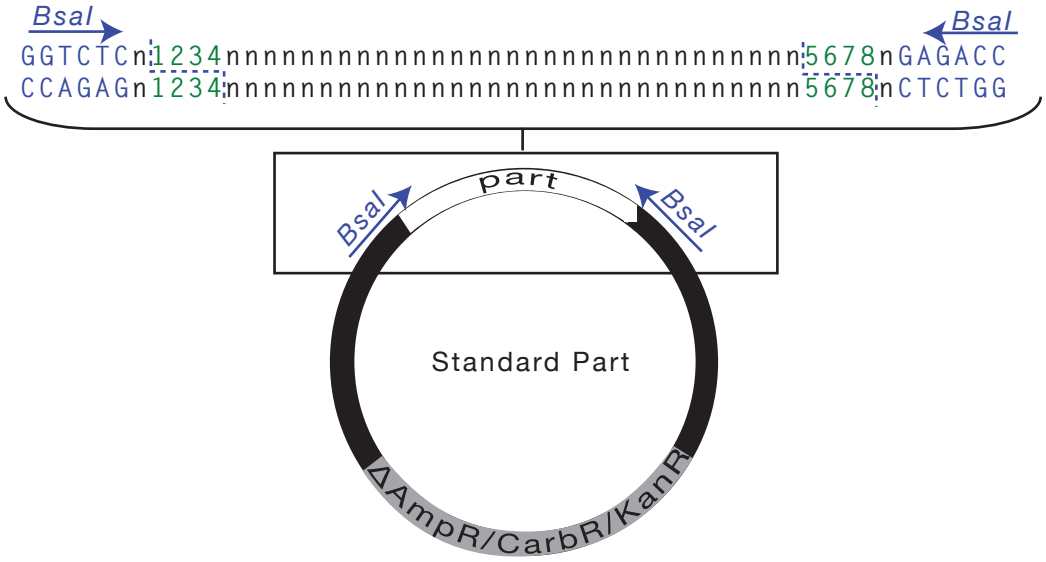
Figure 3 Twelve fusion sites have been defined. These sites allow a multitude of standard parts to be generated. Standard parts comprise any portion of a gene cloned into a plasmid flanked by a convergent pair of *Bsa*I recognition sequences. Parts can comprise the region between an adjacent pair of adjacent fusion sites. Alternatively, to reduce complexity or when a particular functional element is not required, parts can span multiple fusion sites (examples in pink boxes).

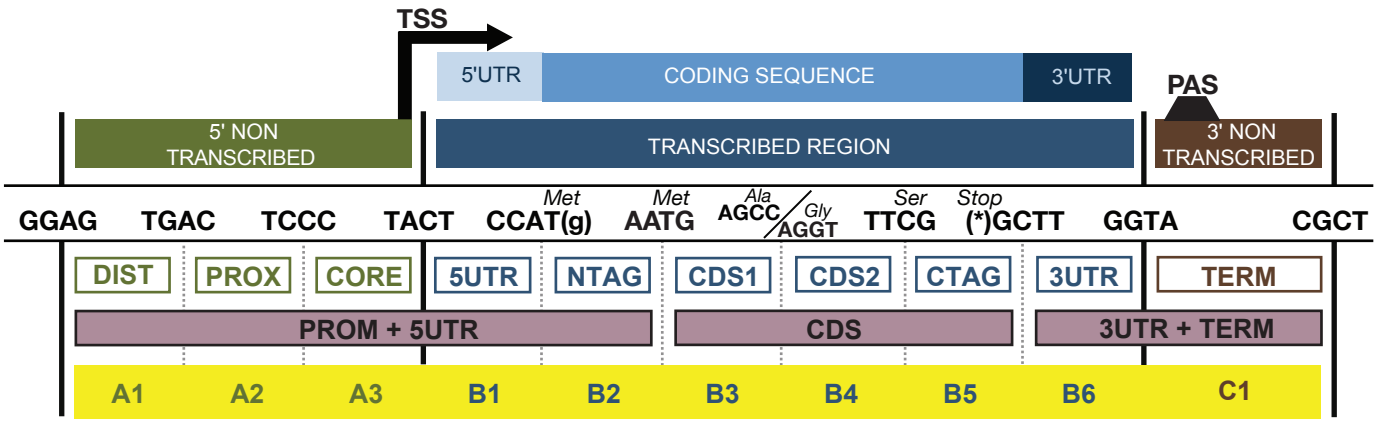
Figure 4 A Universal acceptor plasmids (UAPs) comprise a small plasmid backbone conferring resistance to spectinomycin or chloramphenicol in bacteria. They contain a cloning site consisting of a pair of divergent Type IIS recognition sequences (e.g. *Bpi*I, as depicted, or *Bsm*BI) flanked by overlapping convergent *Bsa*I recognition sequences. **B** A sequence containing an illegal *Bsa*I recognition sequence can be amplified in two fragments using oligonucleotide primers with 5' overhangs (red dashed lines) that (i) introduce a mutation to destroy the illegal site (ii) add TypeIIS recognition sequences (e.g. *Bpi*I, as depicted, or *Bsm*BI) and fusion sites to allow one step digestion-ligation into the universal acceptor and (iii) add the desired fusion sites (green numerals) that will define the type of standard part and that will flank the part when re-released from the backbone with *Bsa*I.



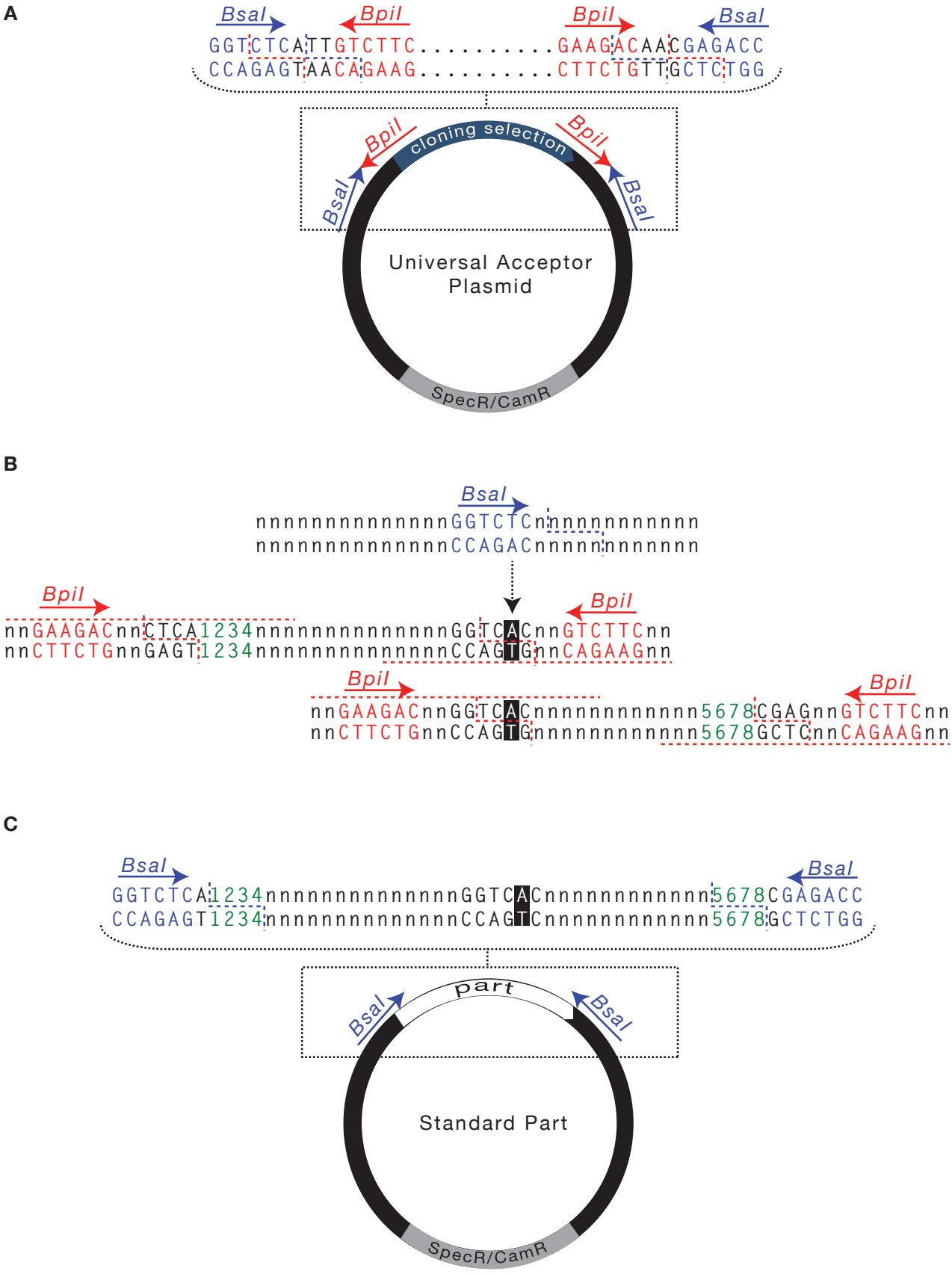
One step digestion-ligation reaction with *BsaI* and T4 ligase.
Selection for colonies carrying plasmids with Bacterial selection B.

TYPE	ENZYME	SEQUENCE	COMPATIBILITY
ILLEGAL	*BsaI*	GGTCTCN:NNNN CCACGAN:NNNN:	Plant Standard MoClo, GB2.0
AVOID	*BpiI*	GAAGACNN:NNNN CTTCTGN:NNNN:	MoClo
AVOID	*BsmBI*	CGTCTCN:NNNN GCAGAGN:NNNN:	GB2.0 MoClo (Level 2i+)





POSITION	NAME	FUNCTION	5' OVERHANG	3' OVERHANG
A1	DIST	Distal promoter region, *cis* regulator or transcriptional enhancer	GGAG	TGAC
A2	PROX	Proximal promoter region, *cis* regulator or transcriptional enhancer	TGAC	TCCC
A3	CORE	Minimal promoter region, including transcription start site (TSS)	TCCC	TACT
A4	5UTR	5' untranslated region	TACT	CCAT
B2	NTAG	N terminal coding region	CCAT	AATG
B3	CDS1	Coding region - optional N terminal coding region	AATG	AGCC /AGGT
B4	CDS2	Coding region - no start or stop codon	AGCC /AGGT	TTCG
B5	CTAG	C terminal coding region	TTCG	GCTT
B6	3UTR	3' untranslated region	GCTT	GGTA
C1	TERM	Transcription terminator including polyadenylation signal (PAS)	GGTA	CGCT


A
BsaI
BpiI
BpiI
BsaI
GGTCTCATTGTCTTC.....GAAGACAACGAGACC
CCAGAGTAACAGAAG.....CTTCTGTTGCTCTGG
BpiI
cloning selection
BpiI
BsaI
BsaI
Universal Acceptor Plasmid
SpecR/CamR
B
BsaI
nnnnnnnnnnnnnnGGTCTCnnnnnnnnnnnn
nnnnnnnnnnnnnnCCAGACnnnnnnnnnnnn
BpiI
BpiI
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BsaI
BsaI
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part
BsaI
BsaI
Standard Part
SpecR/CamR